User Manual

# Lenti-X™ shRNA Expression Systems User Manual

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# I. Introduction

## A. Using Recombinant Lentivirus for shRNA Delivery

Recombinant lentiviruses are powerful, efficient, and versatile vectors for introducing heritable genetic material into the genome of virtually any cell type. Most cells are susceptible to lentivirus infection, including dividing and non-dividing cells, stem cells, and primary cells. (Ausubel *et al.*, 1995; Coffin *et al.*, 1996). The **Lenti-X shRNA Expression System** uses lentivirus to introduce short hairpin RNA (shRNA) expression constructs into cells for the purpose of suppressing the expression of specific genes via RNA interference (RNAi) (Figure 1). To produce infectious lentivirus, the **pLVX-shRNA1 Vector** (Figure 2) containing your shRNA expression sequence, and the **Lenti-X HT Packaging Mix**, are cotransfected into HEK 293T packaging cells using the highly optimized **Lentiphos™ HT** transfection reagents. Viruses accumulate to very high levels in the packaging cell supernatant, which is then used to transduce target cells and initiate RNAi.

- The **pLVX-shRNA2 Vector** (Cat. No. 632179; Figure 2) provides shRNA and fluorescent marker protein coexpression (ZsGreen1), which allows you to easily identify and/or sort transduced cells.
- Clontech has developed several other highly advanced lentiviral expression systems that provide the broad cellular tropism of lentivirus, high viral titers, and excellent transgene expression levels (*Clontechniques*, October 2007).

## B. Inhibiting Gene Expression with shRNAs

shRNA expression is a highly effective strategy for disrupting the function of individual genes in order to study their role in cellular processes. shRNAs containing a user-defined target sequence for any specific gene are easily expressed from a cloned oligonucleotide template, and are a convenient and reproducible means of establishing stable, gene-specific RNAi in mammalian cell lines (Brummelkamp *et al.*, 2002; Paddison *et al.*, 2002; Paul *et al.*, 2002; and Yu *et al.*, 2002). The RNA hairpins trigger endogenous RNAi pathways that respond to the presence of various forms of double-stranded (ds) RNA (for reviews see Hutvagner & Zamore, 2002; Hammond *et al.*, 2001; and Sharp, 2001). Following RNA Pol III transcription, each shRNA adopts a stable stem-loop structure that is further processed to form a ds small interfering RNA (siRNA) that retains the target sequence and guides RNA-induced Silencing Complexes (RISC) in the degradation of the target mRNA.

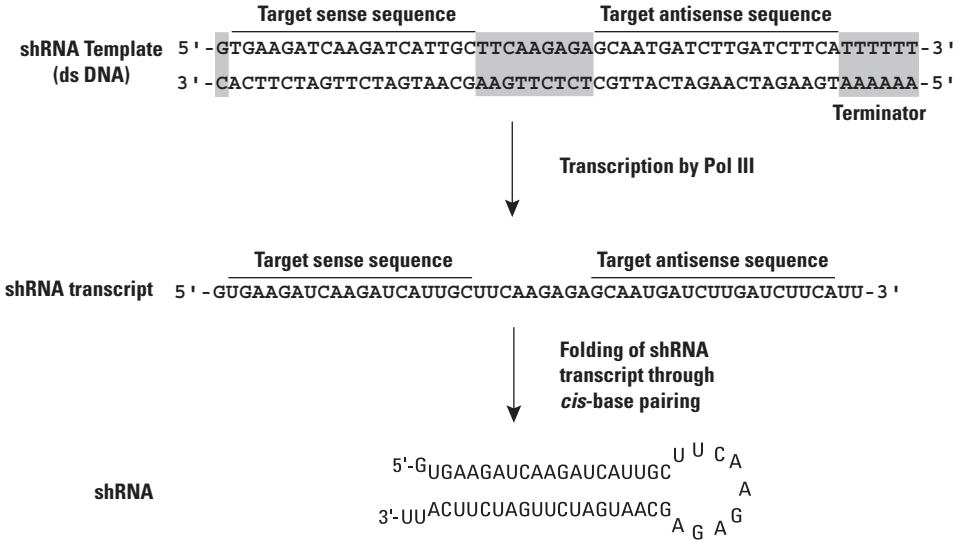


**Figure 1. Small hairpin RNAs (shRNAs) generated from a cloned oligonucleotide DNA template.** This example shows a target sequence derived from the coding region of the ß-actin gene (Harborth *et al.,* 2001). The shRNA-coding oligonucleotides containing the sense and antisense target sequences are cloned downstream of a Pol III promoter in an expression vector designed for gene silencing in mammalian cells. A hairpin loop sequence is located between the sense and antisense sequences on each complementary strand. The transcribed shRNA acts like a ds siRNA molecule and is capable of carrying out gene-specific silencing (Brummelkamp *et al.,* 2002; Paddison *et al.,* 2002; Paul *et al.,* 2002; and Yu *et al.,* 2002).

# I. Introduction continued

## C. Lenti-X Vector Features

Our Lenti-X Expression Vectors (pLVX series) possess lentiviral LTRs and a lentiviral packaging signal (Ψ), as well as other elements that improve transgene expression, viral titer, and overall vector function (Figure 2). A woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) enhances vector packaging by promoting RNA processing events and nuclear export of viral genomic transcripts (Zufferey *et al.,* 1999). In other Lenti-X Systems, the WPRE also boosts transgene expression in target cells by facilitating the production of mature mRNA transcripts (Higashimoto *et al.,* 2007). However, in shRNAs expressed from pLVX-shRNA1, the WPRE is absent from the shRNA precursor because the shRNA cassette contains a Pol III termination sequence. The central polypurine tract/central termination sequence (cPPT/CTS) generates a "DNA flap" which increases nuclear importation of the viral genome during target cell infection, which in turn, results in improved vector integration and transduction (Zennou *et al.*, 2000). The Rev-responsive element (RRE) also helps to increase titers by augmenting the nuclear exportation of unspliced viral genomic RNA (Cochrane *et al.*, 1990).

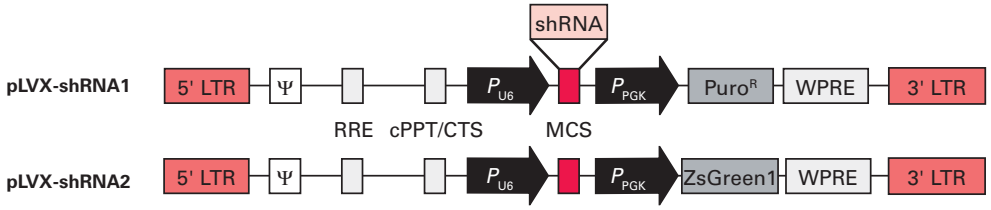


**Figure 2. Clontech's lentiviral pLVX-shRNA vectors for shRNA expression.** Optimum lentiviral vector functions for packaging and shRNA expression are provided by various viral sequence elements and promoters (see text). The pLVX-shRNA1 vector offers puromycin selection capability, while pLVX-shRNA2 provides fluorescent protein coexpression (ZsGreen1).

## D. The Lenti-X HT Packaging System: Safe and Efficient

To produce shRNA lentivirus for infecting target cells, the pLVX-shRNA plasmid is transfected into 293T packaging cells using the **Lenti-X HT Packaging System**, which consists of the Lenti-X HT Packaging Mix and the Lentiphos HT transfection reagents (Figure 3). This system enables you to produce exceptionally high titers of safe, nonreplicating, VSV-G-pseudotyped lentivirus from any lentiviral expression vector.

- **Highest Titers**: The Lenti-X HT Packaging Mix is a plasmid mixture that expresses all the necessary lentiviral packaging components: Pol (RT-IN), Tat, Rev, and Gag, as well as the VSV-G envelope protein, to high levels and in optimized ratios (Wu *et al.*, 2000; *Clontechniques,* October 2007). These proteins combine to form VSV-G pseudotyped virus particles containing the recombinant viral genome transcribed from the cotransfected lentiviral expression vector (Figure 3). The Packaging Mix includes an expression vector for the Tet-Off® transcriptional activator (tTA; Gossen & Bujard, 1992), which drives the expression of specific viral proteins to extra high levels. This optimized expression strategy, combined with high-efficiency Lentiphos HT transfection, results in very high virus titers that are generally 25–50 times higher than other commercially available packaging systems. As a result, Lenti-X HT supernatants can be used directly for most target cell infections, or may require dilution to achieve the desired multiplicity of infection (MOI).

- **Highest Safety:** The split-gene, *trans*-packaging strategy of the Lenti-X HT System also provides a higher level of biosafety because it effectively prevents the production of replication-competent lentivirus, i.e., Lenti-X viruses cannot replicate in, or transfer genetic material from, infected target cells. Thus, these recombinant lentiviruses are essentially "one-way" vectors. Removing essential viral coding sequences from the transfer vector and separating them onto different plasmids, prevents the transfer of these sequences into virus particles, thus rendering them *replication-incompetent*. The minimal sequence homology between the Lenti-X HT Packaging Mix plasmids and the pLVX vectors also prevents the emergence of replication-competence via homologous recombination.

# I. Introduction continued

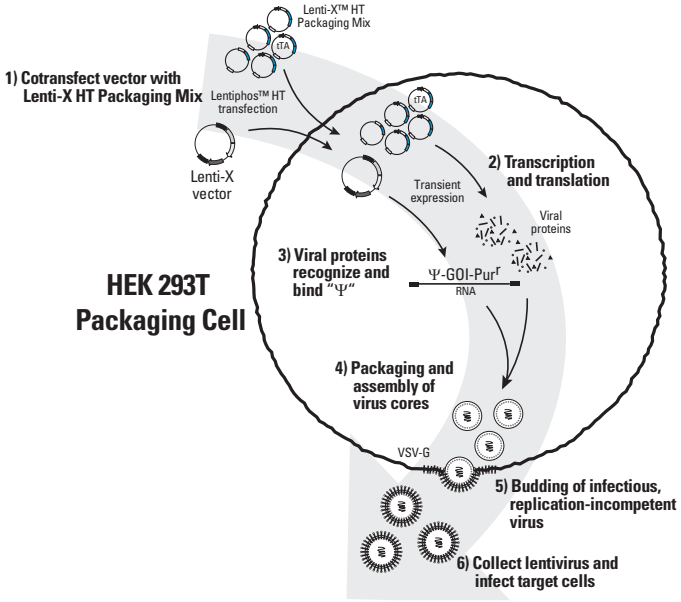


**Figure 3. Lentivirus production using the Lenti-X HT Packaging System and 293T cells.** Initially, cotransfection of a Lenti-X Vector and the Lenti-X HT Packaging Mix (Step 1) results in the production of the corresponding recombinant lentiviral genomic RNA and the required viral packaging proteins (Step 2). A vector in the Packaging Mix expresses the Tet-Off transactivator (tTA) to produce extra-high expression of specific viral components. Recognition of the packaging sequence (Ψ) on the viral RNA by the packaging proteins (Step 3) results in the assembly of virus cores, which are transported to the cell membrane (Step 4). Cores are then enveloped by cellular membrane containing aggregated VSV-G envelope proteins. Mature, infectious virions then bud from the cell (Step 5) and are collected in the medium (Step 6). While infectious, the virions lack several critical genes required for the subsequent replication and production of virus in target cells.

# II. Additional Materials Required

## A. HEK 293T Cells for Lentivirus Packaging

- **HEK 293T cell line:** American Type Culture Collection HEK 293T/17 (ATCC No. CRL-11268™). In order to obtain high-titer supernatants of infectious lentivirus, you will need to transfect your Lenti-X Vectors and the **Lenti-X HT Packaging Mix** into 293T cells using the **Lentiphos HT** transfection system. Together, these components consistently produce very high titers of VSV-G pseudotyped lentivirus.

## B. Lentivirus Titration

- **Lenti-X qRT-PCR Titration Kit (Cat. No. 632165):** This kit provides an extremely fast and simple method for titrating your viral stocks. After a quick RNA purification step, viral genome content is determined using qRT-PCR and SYBR® technologies. The kit works for all HIV-1-based lentiviral vectors and the short 4 hour protocol allows viral harvest and target cell infection to be performed on the same day (Figure 4). You can avoid delays that lead to reduced viral infectivity, and can infect target cells at a known MOI for more consistent results.

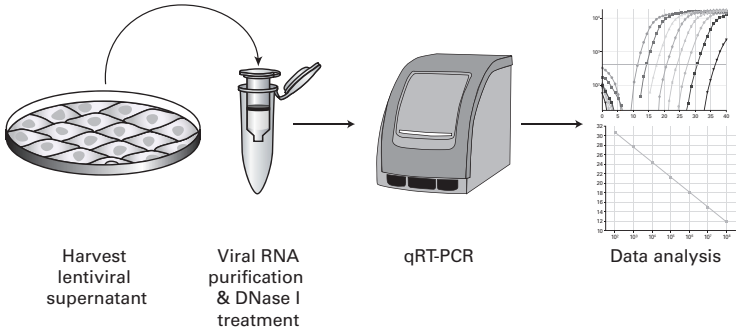


**Figure 4. Flowchart of the procedures used for titering lentiviral supernatants with the Lenti-X qRT-PCR Titration Kit**

# II. Additional Materials Required continued

- **HT-1080 cell line:** American Type Culture Collection HT-1080 (ATCC No. CCL-121™) [Recommended]. For infection-based titrations, this cell line is easily transduced and is frequently used for lentiviral titration. HeLa cells and 293T cells can also be used for virus titration

### C. Mammalian Cell Culture and Transfection Supplies

- **HEK 293T growth medium:** Dulbecco's Modified Eagle's Medium (DMEM) containing 4 mM L-glutamine, 4.5 g/L glucose, 1 mM sodium pyruvate, 3.7 g/L sodium bicarbonate, and 10% fetal bovine serum (tetracycline-free). At Clontech, these cells are cultivated in an incubator containing 5% $CO_2$. Other media formulations and/or $CO_2$/sodium bicarbonate combinations may also be used, but have not been tested by Clontech.
- **HT-1080 growth medium:** Dulbecco's Modified Eagle's Medium (DMEM) containing 4 mM L-glutamine, 4.5 g/L glucose, 1 mM sodium pyruvate, 3.7 g/L sodium bicarbonate, and 10% fetal bovine serum (tetracycline-free). At Clontech, these cells are cultivated in an incubator containing 5% $CO_2$. Other media formulations and/or $CO_2$/sodium bicarbonate combinations may also be used, but have not been tested by Clontech.
- **Tetracycline-free fetal bovine serum** (FBS; see important information below). Serum contaminated with tetracyclines will reduce packaging efficiency, resulting in lower titers. We *strongly recommend* using **Tet System Approved FBS** (Cat. Nos. 631101 & 631106) in all packaging cell culture media.
- Polystyrene culture tubes, 12 x 75 mm (e.g., BD Falcon™ No. 352054) for 293T cell transfections.

### D. Puromycin for Selecting Transduced Cells

Puromycin (Cat. Nos. 631305 & 631306) can be used for selecting target cells transduced with pLVX-shRNA1 and for titrating lentivirus stocks. Potency and cell sensitivity can vary from lot-to-lot, so the optimal selection concentration for each lot and cell type should be titrated as described in Appendix B.

- Recommended working concentration range: 0.25–2 µg/ml (up to 10 µg/ml for certain cell lines)
- Maintenance of stable cell lines: 0.25 µg/ml

**Tetracycline-Free Fetal Bovine Serum (FBS) for Packaging Cell Culture**

Many lots of bovine sera are contaminated with tetracycline (Tc) or Tc derivatives which can be detrimental to Tet Expression Systems (Figure 5). *It is critical to use Tc-free FBS for culturing the 293T packaging cells used with the Lenti-X HT Packaging System.*

- The Lenti-X HT Packaging Mix uses Tet-Off® transactivation to drive high-level expression of specific viral packaging proteins. Tc contaminants in serum will reduce expression of these important components and will negatively affect viral titers.
- We strongly recommend using **Tet System Approved FBS** (Cat. Nos. 631101 & 631106) from Clontech. These sera have been functionally tested in our Tet Systems and found to be free of contaminating Tc activity.

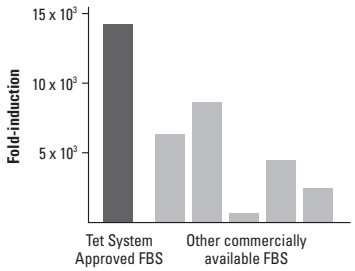


**Figure 5. Tetracycline activity in bovine sera.** The CHO-AA8-Luc Tet-Off Control Cell Line was grown in media prepared with different lots of FBS. Average uninduced expression level = 0.21 RLU (n=21, S.D.=0.07); maximum expression levels varied from 123 to 3,176 RLU.

# II. Additional Materials Required continued

### E. Polybrene for Virus Infection

Polybrene (hexadimethrine bromide; Sigma-Aldrich, Cat. No. H9268) is a polycation that reduces charge repulsion between the virus and the cellular membrane, and facilitates infection of target cells. The optimal polybrene concentration for your target cells (i.e. maximal infectivity with minimal toxicity) should be determined empirically by testing a concentration range of 2–12 µg/ml.

### F. shRNA Oligonucleotides & Materials for Cloning

Since the cloning of shRNA constructs in a pLVX-shRNA vector involves annealing two oligos that are approximately 50-65 nt in length, it is critical that the oligos be highly purified and full-length. Request that your oligos be purified by HPLC for 60-mers, or by PAGE for longer sequences. Finally, it is helpful to verify the length and consistency of your oligos by mass spectrometry. In our opinion, the nominal extra cost for this analysis is well worth it. See Sections VI and Appendix A for guidelines on shRNA target sequence selection and oligonucleotide design.

- T4 DNA Ligase and 10X buffer (e.g., New England Biolabs, Cat. No. M0202S)
- Nuclease-free deionized $H_2O$
- Supercharge™ EZ10 Electrocompetent Cells (Cat. No. 636700)
- Electroporation device for transforming *E. coli*. (e.g., Gene Pulser Xcell System, Bio-Rad Laboratories)

### G. Plasmid DNA Propagation and Purification

For efficient transfections of 293T packaging cells, prepare all plasmids by using NucleoBond or NucleoBond Xtra technology, or by CsCl density gradient purification (Sambrook et al. 2001).

- NucleoSpin® Extract II (Cat. No. 740609.10); for purification of digested pLVX-shRNA1 vector.
- NucleoSpin® Plasmid kit (Cat. No. 740588.10); for mini preparations of plasmid DNA. This DNA should not be used for 293T transfections.
- NucleoBond® Xtra Midi and Maxi kits (Cat. Nos. 740410.10 & 740414.10); for preparation of transfection-quality plasmid DNA.
- NucleoBond® Xtra Maxi EF kit (Cat. No. 740424.10); for preparation of endotoxin-free plasmid DNA.

### H. Gene-Specific Assays

When testing your shRNA construct for functionality, you will need a gene-specific assay to test for the suppression of your target gene. Examples of such assays include:

- Western blotting using an antibody to the protein product
- RT-PCR using specific primers. Ensure that you can discriminate between PCR products generated from mRNA and those derived from genomic DNA
- Northern blot using a gene-specific probe
- Functional assay for the protein product

# III. Safety Guidelines for Working with Lentiviruses

*The protocols in this User Manual require the production, handling, and storage of infectious lentivirus. It is imperative to fully understand the potential hazards of, and necessary precautions for, the laboratory use of lentiviruses.*

The National Institute of Health and Center for Disease Control have designated recombinant lentiviruses as Level 2 organisms. This requires the maintenance of a Biosafety Level 2 facility for work involving this virus and others like it. The VSV-G pseudotyped lentiviruses packaged from the HIV-1-based vectors described here are capable of infecting human cells. The viral supernatants produced by these lentiviral systems could, depending on your insert, contain potentially hazardous recombinant virus. Similar vectors have been approved for human gene therapy trials, attesting to their potential ability to express genes *in vivo*.

For these reasons, due caution must be exercised in the production and handling of any recombinant lentivirus. **The user is strongly advised not to create VSV-G pseudotyped lentiviruses capable of expressing known oncogenes.**

For more information on Biosafety Level 2 agents and practices, download the following reference:

- *Biosafety in Microbiological and Biomedical Laboratories (BMBL),* Fifth Edition (February 2007) HHS Pub. No. (CDC) 93-8395. U.S. Department of Health and Human Services Centers for Disease Control and Prevention and NIH. Available on the web at **http://www.cdc.gov/od/ohs/biosfty/bmbl5/bmbl5toc.htm**

**Biosafety Level 2:** The following is a brief description of Biosafety Level 2. *It is neither detailed nor complete.* Details of the practices, safety equipment, and facilities that combine to produce a Biosafety Level 2 are available in the above publication. If possible, observe and learn the practices described below from someone who has experience working with lentiviruses.

## Important Features of Biosafety Level 2:

- **Practices:**
  - Standard microbiological practices
  - Limited access to work area
  - Biohazard warning signs posted
  - Minimize production of aerosols
  - Decontaminate potentially infectious wastes before disposal
  - Use precautions with sharps (e.g., syringes, blades)
  - Biosafety manual defining any needed waste decontamination or medical surveillance policies
- **Safety equipment:**
  - Biological Safety Cabinet, preferably a Class II BSC/laminar flow hood (with a HEPA microfilter) used for all manipulations of agents that cause splashes or aerosols of infectious materials; exhaust air is unrecirculated
  - PPE: protective laboratory coats, gloves, face protection as needed
- **Facilities:**
  - Autoclave for waste decontamination
  - Chemical disinfectants for spills

# IV. Protocol Overview

***PLEASE READ ALL PROTOCOLS IN THEIR ENTIRETY BEFORE BEGINNING.***

***Successfully using the Lenti-X shRNA Expression System to inhibit the expression of a target gene consists of performing the steps listed below, all of which are described in detail in this user manual.***

1. Select several appropriate mRNA target sequences for your gene of interest (Appendix A). Four sequences should be tested for each target gene.
2. Design and synthesize the shRNA oligonucleotides corresponding to the mRNA target(s) (Section VI).
3. Anneal the shRNA oligos, and clone them into the BamHI/EcoRI-digested pLVX-shRNA1 vector (Section VII).
4. Identify recombinant plasmid clones; propagate and purify the plasmid DNA for transfection (Section VII).
5. Use your recombinant pLVX-shRNA1 vector and the Lenti-X HT Packaging System to produce high-titer lentivirus from 293T packaging cells (Section IX).
6. Titrate your lentiviral supernatant(s) with the Lenti-X qRT-PCR Titration Kit, or by a traditional, infection-based method (Section X).
7. Infect target cells with recombinant lentivirus to express your shRNA and initiate RNAi (Section VIII).
8. Harvest cells for analysis; or select stably transduced, puromycin-resistant clones.

# V. Plasmid DNA Propagation and Purification

## A. General Molecular Biology Techniques

These protocols contain only general information for propagating plasmid vectors and for preparing your pLVX-shRNA Vector. For users requiring more information on standard molecular biology practices and cloning techniques, we recommend the following laboratory references:

- *Current Protocols in Molecular Biology,* ed. by F. M. Ausubel *et al.* (1995, John Wiley & Sons, NY).
- *Molecular Cloning: A Laboratory Manual* ed. by J. Sambrook *et al.* (2001, Cold Spring Harbor Laboratory Press, NY).

## B. Plasmid Vector Propagation & Construction of Your Customized pLVX Vector

1. To ensure that you have a renewable source of plasmid DNA, transform the plasmid vector provided in this kit into an *E. coli* host strain that is recombination deficient and suitable for use with viral vectors (e.g., **Supercharge EZ10 Electrocompetent Cells**, Cat. No. 636756). See the enclosed Vector Information Packet for further DNA propagation details.
2. To generate plasmid DNA for cloning purposes, perform a midi or maxi-scale plasmid prep using a suitable **NucleoBond® or NucleoSpin® Kit.** See **www.clontech.com** for available kits and options.
3. Once your shRNA oligonucleotides have been cloned in the pLVX-shRNA1 Vector, purify a high-quality, transfection-grade, plasmid DNA preparation (midi- or maxi-scale) for each vector that will be transfected into the 293T packaging cells. For guaranteed transfection-grade plasmid DNA, we recommend using **NucleoBond® Xtra Midi Plus** or **Maxi Plus Kits** (Figure 6; Cat. Nos. 740412.10 and 740416.10). For rapid production of *endotoxin-free*, transfection-grade plasmid DNA, use **NucleoBond® Xtra Midi EF Plus** or **Maxi EF Plus Kits** (Cat. Nos. 740422.10 and 740426.10).

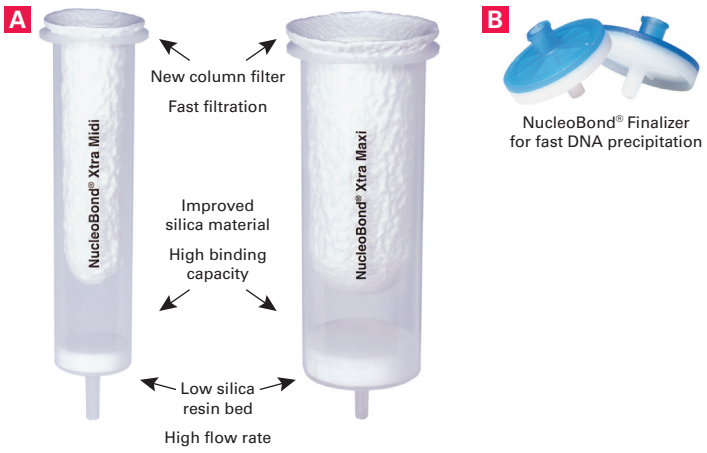


**Figure 6. Advanced features of NucleoBond Xtra Maxi and Midi Columns and NucleoBond Finalizer.** NucleoBond Xtra columns contain a high-flow column filter that minimizes clogging and clears debris from cell lysates during column loading. An improved silica resin provides high DNA-binding capacity, and a wide column diameter keeps the resin bed low for maximum flow rates (**Panel A**). The NucleoBond Finalizer system speeds preparation and increases purity by capturing precipitated DNA on a syringe filter where it can be easily washed and eluted (**Panel B**).

# VI. shRNA Oligonucleotide Design

## A. Selecting shRNA Target Sequences

The degree to which a target gene is knocked down depends largely on choosing ideal target sequence(s) within your gene of interest, and on properly designing the corresponding shRNA oligonucleotides. For users unfamiliar with the requirements of successful mRNA target sequences, we have provided some guidelines for identifying them in Appendix A and in the *"Online Tools"* section of our website (**www.clontech.com**). Further information can be found in Brummelkamp *et al.*, 2002; Paddison *et al.*, 2002; Paul *et al.*, 2002; and Yu *et al.*, 2002. In addition, we highly recommend that you test more than one shRNA sequence per gene of interest (4 sequences are recommended). Depending on the shRNA sequence, the most potent RNAi activity may occur in either the *sense-loop-antisense* configuration or the *antisense-loop-sense* configuration. The hairpin having the strongest inhibitory activity is often determined empirically by testing the shRNA sequence in both orientations.

## B. Design of the shRNA Oligonucleotides

Two complementary oligonucleotides (an upper and lower strand) are needed for each shRNA target site. Figure 7 illustrates the overall structure of the prototypical oligonucleotide sequences that are compatible with the pLVX-shRNA vectors. The oligonucleotide sequences should include the following:

1. A 5'-BamHI restriction site overhang on the upper strand and a 5'-EcoRI restriction site overhang on the lower strand. These restriction sites enable directional cloning of the annealed oligonucleotides into the BamHI/EcoRI-digested pLVX-shRNA1 vector.
2. A guanine (G) residue should be added upstream of the 5'- end of the shRNA sense strand, if the target sequence does not start with a purine, to provide a preferred Pol III transcription start site.
3. The 19-base target sense sequence; see Appendix A for sequence suggestions.
4. A 7–9 nucleotide hairpin loop sequence. (We typically use 5'-TTCAAGAGA- 3'; see Sui *et al.*, 2002; Lee *et al.*, 2002; Paddison *et al.*, 2002; Brummelkamp *et al.*, 2002; and Paul *et al.*, 2002 for other effective loop sequences.)
5. The 19-base target antisense sequence.
6. A RNA Pol III terminator sequence consisting of a 5–6 nucleotide poly(T) tract.
7. Recommended, but not essential: a diagnostic restriction site positioned immediately downstream of the terminator sequence for convenient restriction digest analysis to confirm the presence of the cloned insert. We suggest using Mlu I (5'-ACGCGT- 3') which will generate a ~1.3 kb fragment upon digestion of the recombinant vector, but other sites may work as well.

Thus, beginning at the 5' end, a typical oligonucleotide for the upper strand should have 5 bases to complete the BamHI cloning site, additional G residue (if needed), 19 bases of sense sequence, 7–9 bases of hairpin loop, 19 bases of antisense sequence, 6 bases of terminator T residues, 6 bases of a diagnostic restriction site (MluI), and a final G residue to complete the downstream EcoRI cloning site at the 3' end.

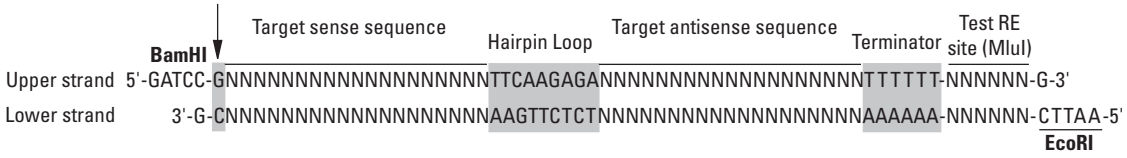


**Figure 7. shRNA oligonucleotide sequence design.** The arrow denotes the purine residue required for RNA Pol III to initiate transcription. The hairpin loop sequence shown is one of many functional loop sequences used to generate shRNAs. Termination is signaled using a poly(T) tract. Including a diagnostic restriction site (Test RE site; i.e. MluI) allows confirmation of the cloned insert after the ligation and transformation reactions. BamHI (upper) and EcoRI (lower) 5' overhangs are necessary for directional cloning into the pLVX-shRNA vector. Visit the "Online Tools" section of our website (www.clontech.com) and see Table II in Appendix A for examples of target sense and antisense sequences for selected genes.

# VI. shRNA Oligonucleotide Design continued

### C. Oligonucleotide Quality

Since cloning your shRNA construct in a pLVX-shRNA1 Vector involves annealing two oligos that are approximately 50-65 nt in length, it is critical that the oligos be highly purified and full-length. Request that your oligos be purified by HPLC for 60-mers, or by PAGE for longer sequences. Finally, it is helpful to verify the length and consistency of your oligos by mass spectrometry. In our opinion, the nominal extra cost for this analysis is well worth it. If the oligonucleotides are to be gel purified, order them at the 200 nmol scale and gel purify them by standard methods. The use of phosphorylated oligonucleotides is not required.

# VII. Cloning shRNA Oligonucleotides in pLVX-shRNA Vectors

**Protocol**
**2-3 hr.**

### A. Protocol: Preparing the pLVX-shRNA Vector for shRNA Oligonucleotide Cloning

The annealed shRNA oligos (Protocol B) will be inserted between the BamHI and EcoRI sites in pLVX-shRNA1. Digestion of the vector with these enzymes liberates a small fragment that is easily removed by spin column purification.

1. Digest 1 µg of pLVX-shRNA1 Vector DNA with BamHI and EcoRI, using the enzyme manufacturer's protocol.
2. Purify the digested vector DNA using a spin column from the NucleoSpin® Extract II Kit (Cat. No. 740609.10), or on an agarose gel using standard methods.
3. Depending on the recovery of digested plasmid, resuspend in 10–20 µl TE buffer (~50 ng/µl).
4. Store the purified vector DNA at –20°C until ready to ligate the annealed oligos.

**Protocol**
**~15 min**

### B. Protocol: Annealing the shRNA Oligonucleotides

For convenience, Steps 3–6 can be performed in a thermal cycler.

1. Resuspend each purified oligonucleotide in TE buffer to a final concentration of 100 µM.
2. Mix the upper strand and lower strand oligos at a ratio of 1:1. This mixture will ultimately yield 50 µM of ds oligo (assuming 100% theoretical annealing).
3. Heat the mixture to 95°C for 30 sec to remove all intramolecular secondary structure and disrupt the internal hairpin of each oligonucleotide. This promotes intermolecular annealing.
4. Heat at 72°C for 2 min.
5. Heat at 37°C for 2 min.
6. Heat at 25°C for 2 min.
7. Store on ice.

The annealed oligonucleotides are now ready for ligation into the pLVX-shRNA1 vector. Alternatively, the annealed oligonucleotides can be stored at –20°C for later use.

# VII. Cloning shRNA Oligonucleotides in pLVX-shRNA Vectors continued

Protocol 1-3 hr.

## C. Protocol: Ligating the Annealed Oligonucleotides into the pLVX-shRNA1 Vector

1. Dilute the annealed oligos (Step B.7) 100-fold with TE buffer to obtain a concentration of 0.5 µM.

   **Note**: To ensure good ligation efficiency, it is necessary to dilute the oligos so that they do not greatly exceed the concentration of the vector DNA. Using a large excess of oligo will inhibit ligation.

2. Assemble a ligation reaction for each annealed pair of oligonucleotides by combining the following reagents in an microfuge tube:

Recipe

| | |
|---|---|
| 1 µl | pLVX-shRNA1 Vector DNA, BamHI/EcoRI-digested (50 ng/µl) |
| 1 µl | Annealed oligonucleotides, diluted (0.5 µM) |
| 1.5 µl | 10X T4 DNA ligase buffer |
| 10.5 µl | Nuclease-free $H_2O$ |
| 1 µl | T4 DNA ligase (400 U/µl) |
| **15 µl** | **Total volume** |

3. If desired, a control ligation can be assembled using 1 µl of nuclease-free $H_2O$ instead of the annealed oligos.
4. Incubate the reaction mixture according to the ligase manufacturer's recommendations.

Protocol ~3 days.

## D. Transform Competent Cells, Identify Recombinant Clones & Prepare DNA for Transfection

**Supercharge EZ10 Electrocompetent Cells** (Cat. No. 636756) provide high transformation efficiency and this strain carries mutations to prevent plasmid DNA rearrangements. These features make EZ10 an ideal host for large plasmids and viral vectors that contain homologous LTR sequences.

1. Transform competent *E. coli* with 2 µl of the ligation reaction, using the protocol supplied with the EZ10 cells.
2. Plate different volumes (20–150 µl) from each transformation on LB agar + ampicillin plates (50–100 µg/ml). Incubate overnight at 37°C
3. Pick 4–8 well isolated colonies from each ligation/transformation and inoculate each into a small-scale liquid culture. Grow overnight at 37°C with shaking.
4. Prepare plasmid DNA minipreps. We recommend using our NucleoSpin® Plasmid Kit (Cat. No. 740588.10).
5. Identify the desired recombinant plasmid by restriction analysis using the diagnostic restriction site within the shRNA oligonucleotide sequence (e.g. MluI, which yields 1.3 kb & 6.5 kb fragments from a recombinant vector). Verify your shRNA insert by sequencing.

   Note

   **Note**: Since there is always a chance for mutations in the oligo due to synthesis errors, we strongly recommend that you sequence at least two clones to verify the correct oligo sequence. Because hairpin sequences are difficult to sequence, inform your sequencing facility so that sequencing conditions can be adjusted accordingly.

6. Once a positive clone has been identified, make a large-scale DNA prep of the recombinant pLVX-shRNA1 vector. To ensure optimal purity of the DNA for transfection, using a NucleoBond® or NucleoBond® Xtra Kit, or CsCl density gradient purification (Sambrook *et al.*, 2001). Do not use miniprep or NucleoSpin-prepared DNA for transfections.

# VIII. Cell Culture Guidelines

## A. General Cell Culture and Lentivirus Information

The protocols in this User Manual provide only general guidelines for lentivirus use and mammalian cell culture techniques. Perform all steps involving cell culture using sterile technique in a Biosafety Level 2 tissue culture hood that has been approved for use with lentiviruses. For users requiring more information on lentiviruses, retroviruses, and mammalian cell culture, we recommend the following general references:

- *Retroviruses,* ed. by J. M. Coffin, S. H. Hughes & H. E. Varmus (1997, Cold Spring Harbor Laboratory Press, NY)
- *Culture of Animal Cells,* 5th Edition, ed. by R. I. Freshney (2005, Wiley-Liss, NY)
- *Current Protocols in Molecular Biology,* ed. by F. M. Ausubel, *et al.* (1995, Wiley & Sons)

**Protocol**
**1 hr**

## B. Protocol: Starting HEK 293T Cultures from Frozen Stock

Frozen cells should be cultured immediately upon receipt, or as soon as possible thereafter. If culturing is significantly delayed after receipt, decreased cell viability may result. For HEK 293-based cell lines, we recommend using collagen-coated plates or flasks for efficient culturing of frozen stocks. Vessels coated with compounds other than collagen may also provide suitable growth substrates (e.g. poly-L-lysine), but only collagen has been tested at Clontech. Once recovered, the cells may be cultured directly on tissue culture plastic. However, if adherence is poor, we recommend using only collagen-coated vessels.

To prevent osmotic shock and maximize cell survival, perform the following:

1. Thaw the vial of cells rapidly in a 37°C water bath with gentle agitation. Immediately upon thawing, wipe the outside of the vial with 70% ethanol. All of the operations from this point on should be carried out in a laminar flow tissue culture hood under strict aseptic conditions. Unscrew the top of the vial slowly and, using a pipet, transfer the contents of the vial to a 15 ml conical centrifuge tube containing 1 ml of pre-warmed medium. Mix gently.
2. Slowly add an additional 4 ml of fresh, pre-warmed medium to the tube and mix gently.
3. Add an additional 5 ml of pre-warmed medium to the tube, mix gently. Centrifuge at 100 x g for 5 min, carefully aspirate the supernatant, and GENTLY resuspend the cells in complete medium. (This method removes the cryopreservative and can be beneficial when resuspending in small volumes. However, be sure to treat the cells gently to prevent damaging fragile cell membranes.)
4. Mix the cell suspension thoroughly and add to a suitable culture vessel. Gently rock or swirl the dish/flask to distribute the cells evenly over the growth surface and place it in a 37°C humidified incubator (5–10% $CO_2$ as appropriate) for 24 hr.
5. The next day, examine the cells under a microscope. If the cells are well-attached and confluent, they can be passaged for use. If the majority of cells are not well-attached, continue culturing for another 24 hr. Complete attachment of newly thawed cultures of HEK 293-based cell lines may require up to 48 hr.

# IX. Producing Lentivirus from pLVX-shRNA Vectors

Protocol 2–3 days

## Protocol: Producing Lentivirus with the Lenti-X HT Packaging System

To obtain the highest titers and the best possible packaging results, adhere strictly to the following protocol, especially with respect to: **(1) culture size and volume; (2) DNA amounts and transfection-grade quality; (3) tetracycline-free serum in 293T growth media; and (4) incubation times**.

All Lentiphos transfection reagents, volumes, and conditions are optimized for use with Lenti-X Vectors, the Lenti-X Packaging Mix, and 293T-based cells. Use 100 mm tissue culture plates and be sure to use **Tet System Approved FBS** (guaranteed Tc-free), both in the transfection medium (Step 1) and in the medium used to collect the virus (Step 8). Tetracycline-contaminated serum is detrimental to the expression of essential packaging components in the Lenti-X Packaging System (see Section II.C).

Attention

*Perform all steps in a sterile tissue culture hood. Lentivirus requires the use of a Biosafety Level 2 facility. The VSV-G pseudotyped lentiviruses packaged from the HIV-1-based vectors described here are capable of infecting human cells. Use appropriate safety precautions (See Section III).*

**STOP!**
**Don't forget:**
- Tet System Approved FBS
- 100 mm culture plates
- Transfection-grade DNA

1. One day before the transfection, plate sufficient 293T cells to achieve 50–80% confluency on the day of the transfection. Generally, we plate 4–5 x $10^6$ cells/100 mm plate in 10 ml of complete growth medium containing Tc-free FBS. Keep the cells in the incubator until just before you are ready to add the transfection mixture (Step 6).
2. In a 12 x 75 mm polystyrene culture tube (e.g., BD Falcon™ No. 352054), add **Lenti-X HT Packaging Mix** (15 µl), your **pLVX-shRNA plasmid DNA** (3 µg) , and sufficient **Sterile $H_2O$** to achieve a final volume of 438 µl. For example:

| | |
|---|---|
| 15 µl | Lenti-X HT Packaging Mix |
| 6 µl | pLVX-shRNA plasmid DNA, (i.e. 3 µg at 0.5 µg/µl) |
| 417 µl | Sterile $H_2O$ |
| **438 µl** | **Total Volume** |

3. Add 62 µl of **Lentiphos1** solution to the DNA solution and vortex thoroughly.
4. While vortexing the DNA/Lentiphos1 solution, add 500 µl of **Lentiphos2,** dropwise into the tube.
5. Incubate at room temperature for 5–10 min to allow the DNA precipitate to form.
6. Remove the plate(s) of 293T cells from the incubator. Gently vortex the transfection solution, and add the entire contents of the tube (1 ml), dropwise, to the cell culture medium.
7. Gently move the plate(s) back and forth to distribute the transfection solution evenly. Incubate the plate(s) at 37°C for 8 hr to overnight in a $CO_2$ incubator. Do not disturb the plates once they are placed in the incubator.
8. After 8 hr to overnight, replace the transfection medium with 10 ml fresh complete growth medium (containing Tc-free FBS) and incubate at 37°C for 24–48 hr. Viral titers will generally be highest at 48 hr after the start of transfection. *Caution: discarded medium contains live lentivirus.*
9. Harvest the lentivirus-containing supernatants. *Caution: supernatants contain live lentivirus.* Pool similar stocks, if desired. Centrifuge briefly (500 x g for 10 min) or filter through a 0.45 µm filter.

   Note

   **Note**: Use only cellulose acetate or polyethersulfone (PES) (low protein binding) filters. Do not use nitrocellulose filters. Nitrocellulose binds surface proteins on the lentiviral envelope and destroys the virus.

10. Either titrate the virus stock (Section X), use the virus to transduce target cells, or freeze the stock in aliquots as described in Step 11.
11. To store the virus stock and avoid multiple freeze-thaw cycles, aliquot the cleared supernatant into single-use cryotubes. Store tubes at –80°C. No cryoprotectant is required.

    **Note**: Titers can drop as much as 2–4 fold with each freeze-thaw cycle (Higashikawa & Chang, 2001; Kwon *et al.*, 2003).

# X. Determining Lentiviral Titer

## A. Summary

To produce consistent transduction results using a known MOI, it is necessary to titrate your lentiviral stocks. You may choose to use the **Lenti-X qRT-PCR Titration Kit** (Cat. No. 632165) for very rapid titrations of virus stocks (~4 hr), or a traditional method that relies on infection. The latter consists of infecting cells with serial dilutions of the stock, selecting for stable transductants with puromycin, and counting the resulting cell colonies (Section B). Alternatively, if you are using the **pLVX-shRNA2 Vector** that contains the ZsGreen1 marker, fluorescence and flow cytometry can be used to determine viral titers. Titers determined using fluorescence are generally higher than those determined by antibiotic selection. Freshly harvested virus can be titered immediately, or frozen in aliquots at –80°C and then titrated. Note that each freeze-thaw cycle can reduce the functional titer of the virus stock by up to 2–4 fold. Absolute titers will depend heavily on the nature of the cells being infected, and there may be significant differences between the titer values obtained for cells commonly used for titration (e.g. HT-1090 cells) and the number of target cells that are ultimately transduced. Nevertheless, titrations serve to quantitate the relative virus content of different viral stocks prepared from different vectors. Determining the viral titer is necessary for three reasons:

- Confirming that the packaging reaction was successful and that viral stocks are viable.
- Allows you to adjust the MOI for your particular cell type and thus control the viral copy number present in transduced cells.
- Determining the maximum number of target cells that can be infected for a given virus volume.

**Protocol 6–11 days**

## B. Protocol: Determining Viral Titer Using Antibiotic Selection

1. On the day before performing the titration infections, plate the cells (e.g. HT-1080 cells) in 6-well plates. Plate 2 x $10^5$ cells/well, in 2 ml of medium. Allow at least one well to be used as a "no infection" control.
2. Prepare 20 ml of complete medium and add 60 µl of 4 mg/ml polybrene. This concentration of polybrene (12 µg/ml) will be diluted 3-fold for a final concentration of 4 µg/ml during transduction.

   **Note**: Polybrene is a polycation that reduces charge repulsion between the virus and the cellular membrane. The optimum final concentration of polybrene may be determined empirically but generally falls within a range of 2–12 µg/ml. Excessive exposure to polybrene (>24 hr) can be toxic to cells.

3. Prepare filtered viral supernatant from packaging cells (Section IX). This is the virus stock.
4. Prepare six 10-fold serial dilutions of the virus stock as follows:
   a. Add 1.35 ml of medium containing polybrene (Step 2) to each of six sterile and numbered 1.5 ml microfuge tubes.
   b. Add 150 µl of the virus stock (Step 3) to tube 1. Mix.
   c. Transfer 150 µl from tube 1 to tube 2 and mix. Continue making serial dilutions by transferring 150 µl from each successive dilution into the next prepared tube.
5. Infect the HT-1080 cells by adding 1 ml of each viral dilution (Step 4) to each appropriate well. The final polybrene concentration will be 4 µg/ml in ~3 ml. Centrifuge the cultures to improve infection efficiency*.
6. After infecting for 8–24 hours, remove supernatants and subject the cells to puromycin selection using the selection concentrations that are optimal for your cell line (see Appendix B).
7. Allow colonies to form for 5–10 days. Stain the colonies with 1% crystal violet solution (in 10% ethanol), and count.
8. The titer value corresponds to the number of colonies generated by the highest dilution, multiplied by that dilution factor. For example, if 4 colonies were produced by the $10^6$ dilution, the viral titer would be 4 x $10^6$ colony forming units (cfu).

***Culture Centrifugation Increases Infection Efficiency**

Centrifuging the plate at 1,200 x g for 60–90 min at 32°C can significantly increase infection efficiency. A room temperature centrifuge is acceptable if a 32°C unit is not available.

# X. Determining Lentiviral Titer continued

## C. Alternative Methods of Virus Titration

- You may also estimate viral titer by infecting HT-1080 cells with virus produced using a control vector which contains an easily detectable reporter gene (e.g. fluorescent protein). Test virus infection on both HT-1080 cells and your target cells. Infecting your target cell line will give you a rough, but rapid, estimation of infection success relative to the HT-1080 cell line. You can use other cell lines to determine viral titer, but HT-1080 cells are widely accepted as the standard target cell for titering lentivirus because these cells are transduced very efficiently. Note that the same virus preparation can yield different "apparent" titers on different cells lines due to variations in receptor expression and transduction efficiency.
- Some variations of the drug-resistance colony assay employ: a shorter selection period (3 days; Byun *et al.*, 1996); recently-infected target cells (Tafuro *et al.*, 1996; Miyao *et al.*, 1995); or *in situ* PCR (PRINS; Claudio *et al.*, 2001).
- Other methods can be use for the direct quantitation of virus particles, including:
  - Slot blots (Nelson *et al.*, 1998; Murdoch, *et al.*, 1997; Onodera *et al.*, 1997)
  - PCR applied to viral supernatants (Quinn & Trevor, 1997; Morgan *et al.*, 1990)
  - Reverse transcriptase activity (Goff *et al.*, 1981).

# XI. Transducing Target Cells with Your shRNA Lentivirus

The following protocol is a general method for transducing adherent cells, such as HT-1080 or HeLa cells. Use it as a starting point for determining the optimal transduction conditions for your target cells. Refer to Appendix C for additional references and alternative infection methods.

Protocol 2–3 days

## A. Protocol: Transducing Target Cells with shRNA Lentivirus

1. Plate target cells in complete growth medium, 12–18 hr before infection.
2. Thaw aliquots of your filtered and titered lentiviral stocks, or use filtered virus stocks freshly prepared from packaging cells (Section IX). Mix gently, but do not vortex. Note that each freeze-thaw cycle will decrease titer by ~2–4-fold.
3. Adjust the volume of the culture medium to accommodate the addition of virus and polybrene. Add sufficient polybrene to obtain the desired final concentration during the transduction step (e.g. 4 µg/ml).

   **Note**: Polybrene is a polycation that reduces charge repulsion between the virus and the cellular membrane. The optimum final concentration of polybrene may be determined empirically but generally falls within a range of 2–12 µg/ml. Excessive exposure to polybrene (>24 hr) can be toxic to cells.

4. Dilute the lentiviral stocks with medium to obtain the desired MOI. If titer values are unknown, use serial dilutions of the virus stock or supernatant such that the total volume of virus represents no more than 1/3 the final volume of culture medium used for transduction. See Information Box below.
5. Add viral supernatant to the cells and transduce for 8–24 hr. Centrifuge the cultures to improve infection efficiency (see Section X.B). If you are concerned that exposing your target cells to either the polybrene or to the viral supernatant (which contains medium conditioned by the packaging cells) may adversely affect their viability, limit the infection to 6–8 hr.
6. Remove and discard the virus-containing transduction medium and replace it with fresh growth medium.
7. Continue to incubate the cells for 24–48 hr to allow the shRNA to achieve its maximum effect. A time course experiment may be necessary in order to determine the optimum time for harvesting the cells.
8. Harvest the cells for analysis or proceed with selection using puromycin (if using LVX-shRNA1 virus).

   **Note**: To determine the efficiency of transduction, you can subject a small subpopulation of cells to antibiotic treatment and harvest the remaining cells for analysis. The cells should be used as soon as possible, but not earlier than 24 hr after transduction.

### Using Untitered Lenti-X HT Stocks and Supernatants

Attention

The Lenti-X HT Packaging System is capable of producing very high titers of virus. Using large excesses of virus can often be detrimental to target cell performance. If the titer of your virus stock is unknown, perform transduction experiments using several different fold-dilutions to test a range of MOIs and get an estimate of viral titer. At Clontech, our scientists are often able to transduce an entire 100 mm dish of target cells using just 10–100 µl of unconcentrated supernatant (Figure 8).

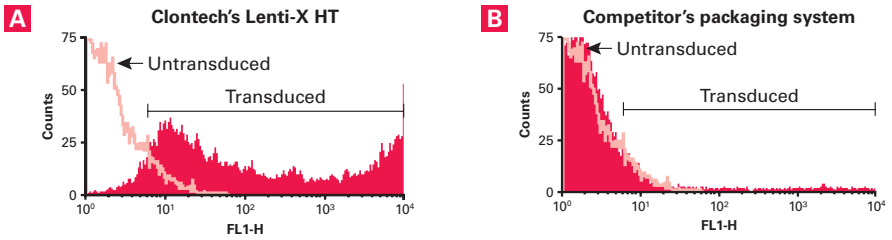


**Figure 8. High infectivity of supernatants produced by the Lenti-X HT Packaging System.** The Lenti-X HT Packaging System (**Panel A**) and a packaging system from a competitor (**Panel B**) were each used to generate viral supernatants from their respective lentiviral system vector that was engineered to express the ZsGreen1 fluorescent protein. As little as 10 µl of supernatant from the Lenti-X HT Packaging System transduced the majority of these HeLa cells, whereas 10 µl of supernatant from the other system transduced only a small percentage of the cells. Transduced cells were quantitated by flow cytometry.

# XII. Troubleshooting Guide

**Table I. Troubleshooting Guide for Lenti-X Expression Systems**

| Description of Problem | Explanation | Solution |
|---|---|---|
| **A. Vector and shRNA Oligonucleotide Cloning** | | |
| Problems with vector growth, stability | Plasmid vectors may undergo rearrangement between the 5′ and 3′ LTRs | Use **Supercharge EZ10 Electrocompetent Cells** (Cat. No. 636756) for high DNA yields and to reduce the possibility of DNA rearrangement. |
| Problems with oligonucleotide cloning | Incompatible ends on the oligos | Confirm that the 5′ ends of the upper and lower annealed shRNA oligos contain BamHI and EcoRI overhangs, respectively. |
| | Ineffective oligo annealing | Verify that the upper and lower strand sequences are correct and complementary. Ensure that equimolar amounts of oligos were used in the annealing reaction. It may be necessary to increase the denaturation temperature prior to slow cooling and annealing. |
| | Oligos are not full-length | Verify oligo size on 12% polyacrylamide gel. Gel purify if necessary, or order HPLC- or gel-purified oligos. Have size verified by mass spectrometry. |
| | Suboptimal oligo concentration in ligation reaction | Verify concentration of the annealed oligos used for ligation. Perform ligations containing 5- to 10-fold range in oligo concentration. |
| | Inactive ligase or buffer | Check ligation reaction with a control vector and fragment. Ligation requires ATP in buffer. |
| **B. 293T Packaging Cells** | | |
| Poor viability upon thawing | Improper thawing techniques | Use thawing procedure in Section VIII.B. |
| | Improper culture medium | Use DMEM with additives listed in Section II.C. Use 10% Tet System Approved FBS (Tc-free). |
| | Improper tissue culture plasticware | Use collagen I-coated plates to aid adherence during initial seeding. |
| Slow growth | Improper culture medium | Use DMEM with additives listed in Section II.C. Use 10% Tet System Approved FBS (Tc-free). |
| Cells do not attach to plate | Improper tissue culture plasticware | Use collagen I-coated plates to aid adherence during initial seeding. |
| Cells appear morphologically different | Passage of cell culture is too high (old cells) | Thaw/purchase new aliquot of 293T cells. |
| **C. Virus Production** | | |
| Poor transfection efficiency | Cell plated too densely | Plate 4–5 x $10^6$ cells/100 mm plate, or fewer if the cells are dividing rapidly. Use at 50–80% confluency. See Section IX. |
| | Transfection is toxic to cells | Use the optimized conditions and reagents as described in Section IX. |
| | Supernatant harvested too soon/too late after 293T transfection | Harvest virus 48 hr after the start of transfection for maximal virus production. |

# XII. Troubleshooting Guide continued

**Table I. Troubleshooting Guide for Lenti-X Expression Systems (continued)**

| Description of Problem | Explanation | Solution |
|---|---|---|
| **C. Virus Production (continued** | | |
| Poor transfection efficiency (cont'd) | Poor DNA quality | Transfection-grade DNA is required. Purify all pLVX-shRNA plasmids using NucleoBond Xtra or Xtra EF. See Section V.B.3. |
| Apparent titer is low (<$10^5$ cfu/ml) | Poor transfection efficiency | See above. |
| | Virus harvested too early | Harvest virus 48–72 hr after start of transfection. |
| | Vector too large (The limit of packaging function is 9.7 kb from 5′-LTR to 3′-LTR) | Concentrate virus for large vectors or reduce size of the insert. See Appendix B for concentration protocol |
| | Polybrene missing or at suboptimal concentration | Add polybrene (4 µg/ml) during transduction or optimize the concentration (4–8 µg/ml) for titration |
| | Virus exposed to multiple freeze-thaw cycles | Each cycle reduces titer by approximately 2–4 fold. Limit the number of freeze-thaws. |
| | If measuring titer by selection, excess puromycin used | Perform an antibiotic kill curve on the cell line prior to using it for titration. |
| | Serum in medium contains tetracycline contaminants | Use Tet System Approved FBS (Cat. No. 631101 & 631106) in the 293T culture medium. |
| | Gene targeted by the shRNA is essential and affecting cell viability | Consider using a Tet-inducible shRNA System to control shRNA expression (see www.clontech.com) |
| **D. Infection of Target Cells** | | |
| Poor infection efficiency | Low titer | See Section C. |
| | Transduction protocol not optimized | See Appendix C for references to help with optimizing transduction protocols. |
| | Low viability of target cells during transduction | Optimize culture conditions for targets prior to infection |
| | | Packaging cell line-conditioned media may be affecting cell growth; dilute viral medium or shorten exposure time to viral supernatant. |
| | | Excessive exposure to polybrene: optimize amount of polybrene (titrate) or shorten exposure time to viral supernatant |
| Infection is toxic to target cells | MOI too high (i.e. too much virus used) | Dilute virus stock; perform titration. |
| | Polybrene toxicity | Reduce or optimize polybrene concentration; reduce infection time. |
| | Packaging cell supernatant or medium is toxic to cells | Dilute virus stock using target cell culture medium; harvest virus from packaging cells using target cell medium |

# XII. Troubleshooting Guide continued

**Table I. Troubleshooting Guide for Lenti-X Expression Systems (continued)**

| Description of Problem | Explanation | Solution |
|---|---|---|
| **E. RNAi Activity** | | |
| Poor knockdown efficiency | Suboptimal mRNA target sequence | Review the target and oligonucleotide requirements described in Appendix A. Test at least 4 shRNA sequences for optimal gene silencing. Large scale functional screening of shRNA sequences is available with Knockout Clone & Confirm PCR Kits. The shRNA sequences so tested can be readily transferable to pLVX-shRNA vectors as BamHI-EcoRI fragments. |
| | Low transduction efficiency | See Section D |
| | Poor target cell viability | Check growth parameters. Use only healthy cells for infection. |
| Target cells dying | Gene targeted by the shRNA is essential | Consider using a Tet-inducible shRNA System to control shRNA expression (see www.clontech.com) |

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# Appendix A: shRNA Target Sequence Requirements

This section describes the features of effective target sequences and their corresponding shRNA oligonucleotides. In addition, Clontech has comprehensive online algorithms to assist you with designing your shRNA oligonucleotides for cloning. Our *RNAi Target Sequence Selector* and *shRNA Sequence Designer* tools can be found in the *"Online Tools"* section of our website (**www.clontech.com**). See Table I for examples of target sequences used to successfully disrupt expression of the cognate genes.

**Note**: The resulting upper and lower strand oligonucleotides should have BamHI and EcoRI 5′-overhangs, respectively, for cloning into pLVX-shRNA vectors.

Select oligonucleotides and target sequences (19 nt) that have the following characteristics:

1. Do not select target sequences within the 5' and 3' untranslated regions (UTRs), nor regions within 75 bases of the start codon. These may be richer in regulatory protein binding sites (Elbashir *et al.,* 2001). UTR-binding proteins and/or translation initiation complexes may interfere with binding of the RISC.
2. Do not select sequences that contain a consecutive run of 3 or more thymidine residues; a poly(T) tract within the sequence can potentially cause premature termination the shRNA transcript.
3. The GC content should be between 40% and 60%; a GC content of approximately 45% is ideal.
4. Sequences that have at least 3 A or T residues in positions 15–19 of the sense sequence may have increased knockdown activity.
5. Each oligonucleotide sequence should have minimal secondary structure (other than the sense-antisense interactions) and be without long base runs, both of which can interfere with proper annealing. Eliminate candidate sequences that display these characteristics.
6. Compare the remaining candidate sequences to an appropriate genome database to identify sequences that are specific for the gene of interest and lack significant homology to other genes. Candidate sequences that meet these criteria are potential shRNA target sites.
7. Test at least 4 shRNAs per gene. It may help to choose shRNA targets that are distributed along the length of the gene sequence to reduce the chance of targeting a region that is either highly structured or bound by regulatory proteins.
8. Depending on the shRNA sequence, the most potent RNAi activity may occur in either the *sense-loop-antisense* configuration or the *antisense-loop-sense* configuration. The hairpin having the strongest inhibitory activity is often determined by testing the shRNA sequence in both orientations.

**Table II. Examples of Published Target Sequences[a]**

| Gene | Target sequence[b] | Sense sequence | Antisense sequence | Reference |
|---|---|---|---|---|
| β-actin | AATGAAGATCAAGATCATTGC | TGAAGATCAAGATCATTGC | GCAATGATCTTGATCTTCA | Harborth *et al.*, 2001 |
| Bcr-abl | AAGCAGAGTTCAAAGCCCTT | GCAGAGTTCAAAGCCCTT | AAGGGCTTTGAACTCTGC | Scherr *et al.*, 2002 |
| hRad9 | AAGTCTTTCCTGTCTGTCTTT | GTCTTTCCTGTCTGTCTTT | AAAGACAGACAGGAAAGAC | Hirai & Wang, 2002 |

[a] Sequences are shown for upper strand oligo design. All sequences are shown 5' to 3'. Lower strand oligo design (not shown) is the complementary sequence to the top strand.
[b] Identified from gene coding sequence.

# Appendix B: Supplemental Protocols

### A. Protocol: Titrating Antibiotics for Selecting Stable Cell Lines.

Prior to using the antibiotics G418 (Cat. No. 631308) and/or puromycin (Cat. No. 631306) to select cells that have been either singly- or doubly-transduced with Lenti-X lentiviruses, it is necessary to titrate each selection agent to determine the optimal concentration for your target cell line. With HeLa cells, for example, we have found 400 µg/ml G418 and 1.0 µg/ml puromycin to be optimal.

- For selecting stable transformants with G418, use the lowest concentration that results in massive cell death in ~5 days and kills all the cells within two weeks.
- Puromycin selection occurs more rapidly; use a concentration that will kill all cells within 3–4 days.
- Lot-to-lot variations in potency exist for all selection drugs, so each new lot of antibiotic should be titrated.

1. For each antibiotic to be tested, plate 2 x $10^5$ cells in each well of a 6-well plate containing 3 ml of the appropriate complete medium plus increasing concentrations of G418 (0, 50, 100, 200, 400, and 800 µg/ml). For puromycin, add the drug at 0, 1.0, 2.5, 5.0, 7.5, and 10.0 µg/ml.
2. For G418, incubate the cells for 5–10 days or until all cells are dead. Examine the dishes for viable cells every two days. Replace the selective medium every four days (or more often if necessary), until the optimal concentration is determined.
3. For puromycin, incubate the cells 4–7 days. Replace medium after 2 days to remove dead cells.

### B. Protocol: Concentrating Virus by Ultracentrifugation

This should be used for VSV-G-enveloped virions only (Burns et al, 1994).

1. Remove cell debris and aggregated virus by low speed centrifugation (500 x g) for 10 min at 4°C.
2. Using an ultracentrifuge, pellet the virus at 50,000 x g for 90 min at 4°C. Remove the supernatant.
3. Resuspend the virus to 0.5–1% of the original volume in TNE (50 mM Tris-HCl [pH 7.8], 130 mM NaCl, 1 mM EDTA), and incubate overnight at 4°C.

   **Note**: If desired, perform a second round of ultracentrifugation (Steps 1–2).

4. Determine the viral titers of pre- and post-concentrated viral supernatants.
5. Transduce target cells as described in Section XI.

# Appendix C: Additional Viral Infection Methods

These references are provided for fine-tuning your transduction protocols so that you may improve your transduction efficiency in target cells. This list is not comprehensive, but many of these protocols will work for a wide range of cell types. You must determine which methods work best for your target cells, and certain methods may be combined for additive effects. For optimization experiments, we recommend using pLVX-shRNA2, or one of our Lenti-X Fluorescent Vectors, which will express a Living Colors Fluorescent Protein. This greatly simplifies the detection and quantitation of lentiviral gene transfer efficiency.

### A. Transduction of cells at 32°C. Decreasing temperature increases viral half-life during transduction.

Bunnell, B. A., Muul, L. M., Donahue, R. E., Blaese, R. M., Morgan, R. A. (1995) High-efficiency retroviral-mediated gene transfer into human and nonhuman primate peripheral blood lymphocytes. *Proc. Natl. Acad. Sci. USA* **92**(17):7739–7743.

Zhou, P., Lee, J., Moore, P., Brasky, K. M. (2001) High-efficiency gene transfer into rhesus macaque primary T lymphocytes by combining 32 degrees C centrifugation and CH-296-coated plates: effect of gene transfer protocol on T cell homing receptor expression. *Hum. Gene Ther.* **12**(15):1843–1855.

Kotani, H., Newton, P. B. 3rd, Zhang, S., Chiang, Y. L., Otto, E., Weaver, L., Blaese, R. M., Anderson, W. F. & McGarrity, G. J. (1994) Improved methods of retroviral vector transduction and production for gene therapy. *Hum. Gene Ther.* **5**(1):19–28.

Higashikawa, F. & Chang, L. (2001) Kinetic analyses of stability of simple and complex retroviral vectors. *Virology* **280**(1):124–131.

### B. Centrifugation during transduction ("spinoculation"), may counteract diffusion of virus when binding target cells

Bunnell, B. A., Muul, L. M., Donahue, R. E., Blaese, R. M. & Morgan, R. A. (1995) High-efficiency retroviral-mediated gene transfer into human and nonhuman primate peripheral blood lymphocytes. *Proc. Natl. Acad. Sci. USA* **92**(17):7739–7743.

Ohkubo, T., Barcena, A., Smith, C. A., Harrison, M. R. & Muench, M. O. (2001) High-efficiency retroviral transduction of fetal liver CD38-CD34++ cells: implications for *in utero* and *ex utero* gene therapy. *Fetal Diagn. Ther.* **16**(5):299–307.

Movassagh, M., Boyer, O., Burland, M. C., Leclercq, V., Klatzmann, D. & Lemoine F. M. (2000) Retrovirus-mediated gene transfer into T cells: 95% transduction efficiency without further *in vitro* selection. *Hum. Gene Ther.* **11**(8):1189–1200.

Bahnson, A. B., Dunigan, J. T., Baysal, B. E., Mohney, T., Atchison, R. W., Nimgaonkar, M. T., Ball, E. D. & Barranger, J. A. (1995) Centrifugal enhancement of retroviral mediated gene transfer. *J. Virol. Methods* **54**(2–3):131–143.

### C. Precipitation of virus to increase titer (concentration)

Pham, L., Ye, H., Cosset, F. L., Russell, S. J. & Peng, K. W. (2001) Concentration of viral vectors by coprecipitation with calcium phosphate. *J. Gene Med.* **3**(2):188–194.

Darling, D., Hughes, C., Galea-Lauri, J., Gaken, J., Trayner, I. D., Kuiper, M. & Farzaneh, F. (2000) Low-speed centrifugation of retroviral vectors absorbed to a particulate substrate: a highly effective means of enhancing retroviral titer. *Gene Ther.* **7**(11):914–923.

Hughes, C., Galea-Lauri, J., Farzaneh, F. & Darling, D. (2001) Streptavidin paramagnetic particles provide a choice of three affinity-based capture and magnetic concentration strategies for retroviral vectors. *Mol. Ther.* **3**(4):623–630.

# Appendix C: Additional Viral Infection Methods continued

### D. Precipitation, during transduction, facilitates greater contact between the target cells and virions

Le Doux, J. M., Landazuri, N., Yarmush, M. L. & Morgan, J. R. (2001) Complexation of retrovirus with cationic and anionic polymers increases the efficiency of gene transfer. *Hum. Gene Ther.* **12**(13):1611–1621.

Morling, F. J. & Russell, S. J. (1995) Enhanced transduction efficiency of retroviral vectors coprecipitated with calcium phosphate. *Gene Ther.* **2**(7):504–508.

Hennemann, B., Chuo, J. Y., Schley, P. D., Lambie, K., Humphries, R. K. & Eaves, C. J. (2000) High-efficiency retroviral transduction of mammalian cells on positively charged surfaces. *Hum. Gene Ther.* **11**(1):43–51.

### E. Flow through transduction: concentrating cells and virus together in small culture systems

Pan, D., Shankar, R., Stroncek, D. F. & Whitley, C. B. (1999) Combined ultrafiltration-transduction in a hollow-fiber bioreactor facilitates retrovirus-mediated gene transfer into peripheral blood lymphocytes from patients with mucopolysaccharidosis type II. *Hum. Gene Ther.* **10**(17):2799–2810.

Chuck, A. S. & Palsson, B. O. (1996) Consistent and high rates of gene transfer can be obtained using flow-through transduction over a wide range of retroviral titers. *Hum. Gene Ther*. **7**(6):743–750.

### F. Addition of fibronectin: adhesion domains within fibronectin allow binding to both target cells and virions to facilitate colocalization

Zhou, P., Lee, J., Moore, P. & Brasky, K. M. (2001) High-efficiency gene transfer into rhesus macaque primary T lymphocytes by combining 32 degrees C centrifugation and CH-296-coated plates: effect of gene transfer protocol on T cell homing receptor expression. *Hum. Gene Ther.* **12**(15):1843–1855.

Moritz, T., Dutt, P., Xiao, X., Carstanjen, D., Vik, T., Hanenberg, H. & Williams D. A. (1996) Fibronectin improves transduction of reconstituting hematopoietic stem cells by retroviral vectors: evidence of direct viral binding to chymotryptic carboxy-terminal fragments. *Blood* **88**(3):855–862.

Hanenberg, H., Xiao, X. L., Dilloo, D., Hashino, K., Kato, I. & Williams, D. A. (1996) Colocalization of retrovirus and target cells on specific fibronectin fragments increases genetic transduction of mammalian cells. *Nat. Med.* **2**(8):876–882.

Bajaj, B., Lei, P. & Andreadis, S. T. (2001) High efficiencies of gene transfer with immobilized recombinant retrovirus: kinetics and optimization. *Biotechnol. Prog.* **17**(4):587–596.

### G. Cocultivation of target cells and packaging cells: Allows targets to be continuously in contact with freshly-produced viral supernatant

Casal, M. L. & Wolfe, J. H. (1997) Amphotropic and ecotropic retroviral vector viruses transduce midgestational murine fetal liver cells in a dual-chambered cocultivation system. *Gene Ther.* **4**(1):39–44.

Germeraad, W. T., Asami, N., Fujimoto, S., Mazda, O. & Katsura, Y. (1994) Efficient retrovirus-mediated gene transduction into murine hematopoietic stem cells and long-lasting expression using a transwell coculture system. *Blood* **84**(3):780–788.

### H. Use of cationic liposomes: Enhance virus-to-cell fusion

Kaneko, Y. & Tsukamoto, A. (1996) Cationic liposomes enhance retrovirus-mediated multinucleated cell formation and retroviral transduction. *Cancer Lett.* **105**(1):39–44.

Porter, C. D., Lukacs, K. V., Box, G., Takeuchi, Y. & Collins, M. K. (1998) Cationic liposomes enhance the rate of transduction by a recombinant retroviral vector in vitro and in vivo. *J. Virol.* **72**(6):4832–4840.

# Appendix B: Additional Viral Infection Methods continued

### I. Use of histone deacetylase inhibitors to increase titer: Relieves repression of viral expression by hyperacetylation of histones

Chen, W. Y., Bailey, E. C., McCune, S. L., Dong, J. Y. & Townes, T. M. (1997) Reactivation of silenced, virally transduced genes by inhibitors of histone deacetylase. *Proc. Natl. Acad. Sci. USA* **94**(11):5798–5803.

Tobias, C. A., Kim, D. & Fischer, I. (2000) Improved recombinant retroviral titers utilizing trichostatin A. *Biotechniques* **29**(4):884–890.

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